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Kinetics of the Reduction of Metalloproteins by Chromous Ion

(laccase/cytochrome *c*/plastocyanins/temperature/rate constants)

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ABSTRACT **The reduction of Cu(330) in *Rhus vernicifera* laccase by chromous ion is 30% faster than reduction of Cu(614) at room temperature [pH 4.8, μ = 0.1 (NaCl)], and two parallel first-order paths, attributed to heterogeneity of the protein, are observed at both wavelengths. The reactions of stellacyanin, spinach and French-bean plastocyanins, and cytochrome *c* with chromous ion under similar conditions are faster than that with laccase by factors of 10^2 to 10^4, and are first order in protein concentration. Comparison of rates and activation parameters for the reduction of "blue" copper in laccase, stellacyanin, and the two plastocyanins indicates that reduction of the Cu(614) site in laccase may occur by intramolecular electron transfer from one of the Cu(330) sites. Our value of $\Delta H^{\ddagger}$ (17.4 kcal/mol) for the chromous ion reduction of cytochrome *c* is consistent with a mechanism in which major conformational changes in the protein must accompany electron transfer.**

We have begun an extensive investigation of the mechanisms of the reduction/oxidation (redox) reactions between metalloproteins and inorganic inner- and outer-sphere reducing agents. Our initial research has been concerned with the reduction rates of horse heart cytochrome *c* and the copper proteins *Rhus vernicifera* laccase, stellacyanin, and plastocyanins from spinach and French-bean. Cytochrome *c* is particularly valuable as a model protein oxidant because the molecular structures of both oxidized and reduced forms have recently been determined (1, 2). Although as yet no direct x-ray structural data are available for the copper proteins, extensive spectroscopic studies have characterized the different types of Cu^{2+} sites (3).

This paper compares the kinetic behavior of cytochrome *c* and the copper proteins with aqueous chromium(II). These reactions should yield valuable mechanistic information because there is substantial literature on Cr(II) reductions of model transition metal complexes (4). Furthermore, Kowalsky has shown that some of the Cr(III) product remains bound to the protein after the chromous reduction of cytochrome *c* (5). Thus, the substitution-inert Cr(III) may label one or more key reduction sites in reactions with cytochrome *c* and possibly the copper proteins.

MATERIALS AND METHODS

Distilled deionized water was used for kinetics experiments. Concentrated Cr(II) solutions were made up from high-purity Cr metal (Alfa Inorganics) and a slight excess of deoxygenated hydrochloric acid. Deoxygenation was accomplished by purging solutions with nitrogen, which had been passed through towers containing chromous solutions to remove oxidizing impurities. Dilute chromous solutions were prepared by standard syringe techniques and were continuously purged with nitrogen. These solutions were assayed by reacting aliquots with excess standard $KMnO_4$ in 0.2 M H_2SO_4 and determining residual MnO_4^- spectrophotometrically at 545 nm (ϵ_{max} = 2340 M^{-1} cm^{-1}). Analyses for total chromium by the basic peroxide procedure (6) agreed well with results of Cr(II) determinations.

Reagent grade sodium chloride was used to supplement the ionic strength of chromous and protein solutions to 0.1.

Plastocyanins from spinach and French-bean (*Phaseolus vulgaris*, var. Prince) were isolated and purified by modifications of the method used by Katoh *et al.*, (7) for spinach plastocyanin. The chloroplasts were obtained with a Waring blendor rather than by grinding in a mortar, and the purification procedure as improved by gel filtration (Bio-Gel P-60 or P-30) of protein solutions. Samples used for kinetics experiments had A_{278}/A_{597} ratios of 1.24 (spinach) and 1.48 (bean). Stellacyanin and laccase were extracted from the latex of the Japanese lacquer tree *Rhus vernicifera* using the method of Reinhammar (8); the A_{280}/A_{604} ratio (stellacyanin) was 5.6 and the A_{280}/A_{614} ratio (laccase) was 15.3. The horse-heart cytochrome *c* was from the same sample used for the x-ray structural determination (1), and was kindly donated by Dr. T. Takano.

After removal from liquid-nitrogen storage, the copper protein solutions used in this work were dialyzed for 6 hr against two changes of deionized water; they were diluted to yield an absorbance change at about 600 nm of 0.15–0.30 (2-cm path) on reduction.

The pH of protein solutions was adjusted with dilute HCl and a Corning model 12 pH meter before degassing. The pH of dilute chromous solutions was monitored continuously with the pH meter and a Thomas combination electrode, which was inserted through a rubber serum stopper in one neck of the storage flask. Deoxygenated dilute NaOH solution, injected with a syringe, was used to bring Cr(II) solutions to the desired pH.

Chromous reductions of "blue" copper proteins and of cytochrome *c* were followed spectrophotometrically with a Durrum-Gibson stopped flow apparatus. The copper absorption maxima at 597, 604, and 614 nm were used to study the reduction of the two plastocyanins, stellacyanin, and laccase, respectively. Data for *Rhus* laccase were also collected at 330

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nm. The production of ferrocytochrome *c* was observed at 550 nm.

Chromous and protein solutions were transferred from storage flasks directly into the air-tight, stopped-flow syringes with Kel-F and Teflon fittings (Hamilton Co., Whittier, Calif.) and stainless-steel hypodermic needles. The stopped-flow syringes were immersed in a small bath containing water circulated from a constant temperature reservoir (Forma Scientific). Temperature control was estimated to be ±0.2°C.

Pseudo first-order conditions for the metalloproteins were used, a 50- to 100-fold excess of reducing agent being present in most experiments ($[Cr(II)]_0 \cong 5 \times 10^{-3}$ M). Stopped-flow traces were analyzed through the usual plots of log $(A_t - A_\infty)$ against time. Rate constants were evaluated assuming a first-order rate dependence on chromous ion; this assumption will be tested in experiments presently underway.

RESULTS

As shown in Fig. 1, the reduction of cytochrome *c* by an excess of Cr(II) follows a pseudo first-order rate dependence through more than 90% of the reaction. This plot is typical of the data obtained at all temperatures and for all of the metalloproteins studied here, except *Rhus* laccase.

By contrast, the reduction of the "blue" copper of laccase (614 nm) does not follow simple first-order kinetics (Fig. 2). Plots of log $(A_t - A_\infty)$ against time were analyzed by extrapolation of the linear portion of the curve, from the latter part of the reaction, back to the ordinate. The difference between this extrapolated line and the observed curve was plotted on a semilog scale. The consistent linearity of this secondary plot is in accord with a mechanism in which the loss of absorbance at 614 nm is the result of two parallel first-order processes. Laccase data at 330 nm also show two rate

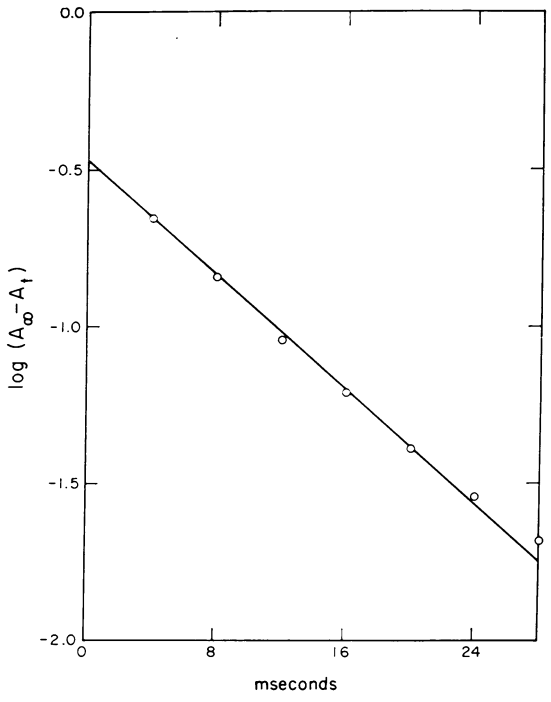

FIG. 1. Typical plot of log $(A_\infty - A_t)$ against time for reduction of cytochrome *c* by chromous ion. T = 37.8°C, (pH 4.2), $[Cr(II)]_0 = 5.4 \times 10^{-3}$ M.

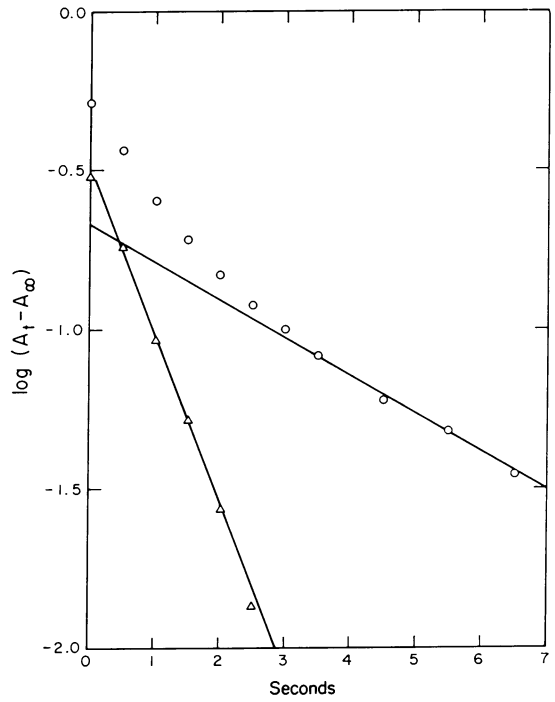

FIG. 2. Plot of log $(A_t - A_\infty)$ against time for the reduction of *Rhus vernicifera* laccase by chromous ion. T = 40.2°C, (pH 4.8), $[Cr(II)]_0 = 5.4 \times 10^{-3}$ M. Reaction followed at 614 nm. *Triangles* mark the derivative plot of log $(A_t - A_\infty)$ for the fast initial phase of the reaction.

constants above 23°C, but at lower temperatures there was no apparent deviation from the simple first-order line.

By comparing the oridnate intercepts of the lines corresponding to fast and slow rate constants with the total change in absorbance from fully oxidized to fully reduced protein, it was possible to estimate that throughout the temperature range the fast-reducing component was responsible for the loss of about two-thirds of the total blue color at 614 nm.

The rate data for the chromous reduction of cytochrome *c* at pH 4.2 are shown in Fig. 3*a* as an Eyring plot of log (k/T) against $1/T$. Thermodynamic activation parameters calculated for this and all other reactions are summarized in Table 1, along with rate constants at room temperature estimated from Eyring plots.

Eyring plots for the chromous ion reduction of laccase at pH 4.8 are shown in Fig. 3*b*. The data for the slow reactions at 614 and 330 nm are so similar that, to within the limits of experimental error, they might be attributed to the same reaction. The fast reaction data at 614 nm at the low temperature extreme consistently deviate from the straight line plot of log (k/T) against $1/T$ plot that best fits all the other points. Further study of this anomaly is necessary.

Spinach and bean plastocyanins showed rate constants of the order of 10^4 M^{-1} sec^{-1}, with little variation with temperature. The bean plastocyanin data show considerable scatter, but no consistent trend with temperature. Consecutive traces obtained at 31.80°C and 4.0°C show less than a 5% difference in rate. We estimate $\Delta H^\ddagger$ to be less than 1 kcal/mol.

Because stellacyanin reacts so rapidly with Cr(II), it was necessary to work with low concentrations of chromous ion

TABLE 1. *Rate data for the chromium(II) reduction of metalloproteins**

Protein	k (25°C) M^{-1} sec^{-1}	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (entropy units)	pH
Cytochrome *c*	1.2×10^4	17.4	+18	4.2
Rhus laccase				
614 nm-				
Fast	24	12.9	−9	4.8
Slow	7	13.8	−9	4.8
330 nm-				
Fast	32	13.4	−7	4.8
Slow	5	15.2	−4	4.8
Spinach plastocyanin	3.3×10^4	1.3	−34	4.2
Bean plastocyanin	1×10^4	~1	—	4.2
Stellacyanin	6×10^5	—	—	4.2

* μ = 0.1 (NaCl); estimated uncertainties are ±1 kcal/mol in $\Delta H^{\ddagger}$ and ±2 entropy units in $\Delta S^{\ddagger}$.

and to use the shortest time scale of the instrument. A second-order rate constant of 6×10^5 M^{-1} sec^{-1} (4°C, pH 4.2) was estimated, and little increase was noted at 20°C.

DISCUSSION

Study of the temperature dependence of cytochrome *c* reductions by small molecules has shown that the hydroquinone reduction is sensitive to pH and ionic strength, with E_a = 20 kcal/mol, $\Delta S^{\ddagger} \cong +15$ entropy units [k = 3.7 M^{-1} sec^{-1}, 12°C (pH 7.0); μ = 0.17] (9). Reduction of cytochrome *c* by the semiquinone radical is about 10^5-times faster. A nonlinear Arrhenius plot has been found for the reduction of cytochrome *c* by ascorbate (10); the slope of the lower temperature region corresponds to E_a = 25–30 kcal/mol (pH 7.0, 0.1 M phosphate buffer). By contrast, the rate of reduction of the "low-pH form" of cytochrome *c* by ascorbate was found to be almost temperature independent. A slower reacting form appeared to be converted to an active form, at a rate characterized by E_a = +19 kcal/mol (11). Very fast reductions of cytochrome *c* by tetrachlorohydroquinone ($k = 6 \times 10^6$ M^{-1} sec^{-1}) (9) and by hydrated electrons (12) have been reported. The ferrocyanide reduction of cytochrome *c* proceeds at a rate very similar to that observed for the chromous reduction (13).

The pulsed radiolysis work of Land and Swallow (12) showed that transfer of an electron to the iron atom of cytochrome *c* is accompanied by the full change in absorbance at 550 nm. The final change in absorbance at shorter wavelengths is completed much more slowly, indicating that after the initial reduction of iron the protein accommodates itself to the new oxidation state in a reaction significantly slower than electron transfer. This secondary reaction was found to have an activation energy of 20 kcal/mol, which is the same as that found for the reduction of cytochrome *c* by hydroquinone and similar to our value of $\Delta H^{\ddagger}$ (17.4 kcal/mol) for the reduction by chromous ion.

It seems possible that with less reactive species than hydrated electrons, electron transfer cannot take place without simultaneous conformational changes in the protein. Such a mechanism would explain the similarity of activation energy terms for various reducing agents. Less efficient reducing agents, of course, might have additional barriers to reaction, but results with other facile one-electron transition metal reductants should clarify whether conformational changes in the protein are rate-determining in the electron transfer reactions of cytochrome *c*.

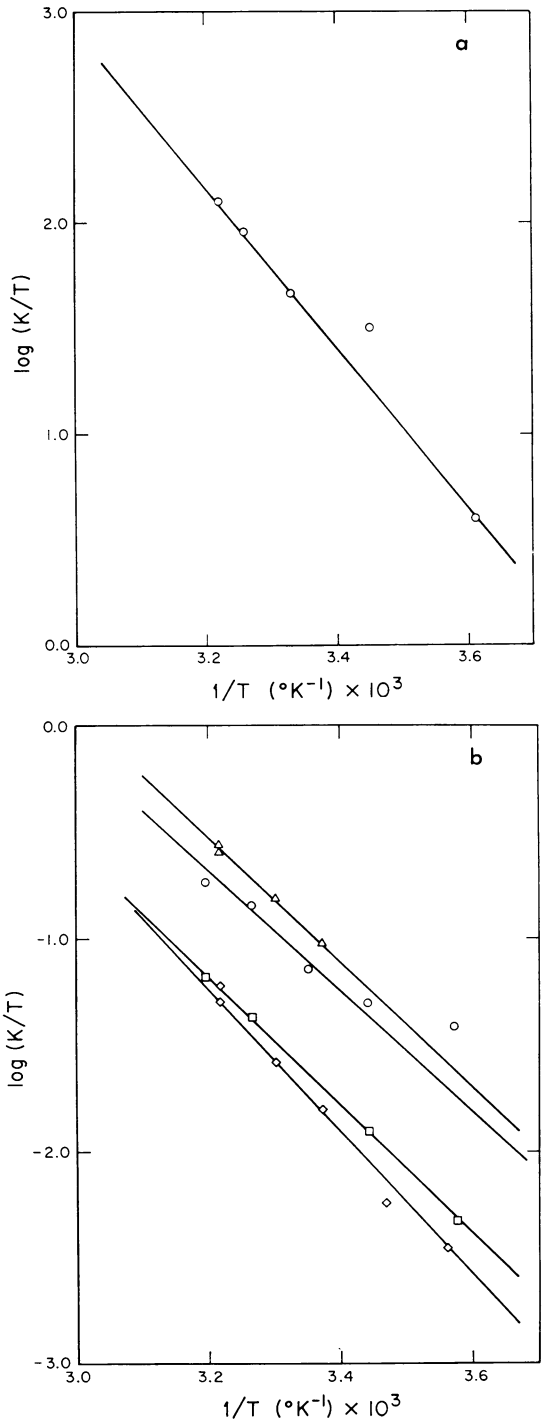

FIG. 3. (*a*) Eyring plot of rate data for the chromous ion reduction of cytochrome *c* at pH 4.2, μ = 0.1.

(*b*) Eyring plots of rate data for the chromous ion reduction of *Rhus vernicifera* laccase at pH 4.8, μ = 0.1. Δ– – –, fast reaction, 330 nm; O– – –, fast reaction, 614 nm; ◇– – –, slow reaction, 330 nm; □– – –, slow reaction, 614 nm.

The unusually favorable $\Delta S^{\ddagger}$ value for the chromous ion reduction of cytochrome *c* is similar to that calculated for the reaction with hydroquinone (9). Such a large positive $\Delta S^{\ddagger}$ is remarkable in view of the negative contribution expected (14) from the solvation changes associated with formation of a transition state involving the strongly basic ferricytochrome *c* with a dipositive ion at pH 4.2. One possible explanation is that the protein conformational change required to reach the transition state places the hydrophobic side chain of phenylalanine 82 substantially into the heme crevice, where it is found in the reduced protein (2), thereby breaking up a significant amount of ordered water structure.

Most studies (3) of laccase have used the protein derived from *Polyporus versicolor*, rather than from *Rhus vernicifera*. The *Polyporus* enzyme has four copper atoms; one is responsible for the 614-nm absorption ("Type 1"), one is detected only from its electron paramagnetic resonance (EPR) spectrum ("Type 2"), and two others ("Type 3" copper), unobservable in EPR spectra, are responsible for the absorption shoulder at 330 nm. There is evidence for cooperative behavior among the copper atoms of this protein; it appears that ion binding at the Type-2 copper inhibits enzymatic activity. Quinol reduces Type-1 copper most rapidly, whereas azide reduces the Type-2 copper first (15). Redox titrations of the molecule show that in the absence of an inhibitor, Type-1 and Type-3 copper atoms are reduced simultaneously.

The *Rhus vernicifera* laccase has only recently been given considerable attention. In general it seems very similar to the fungal enzyme (16). Titration of oxidized laccase by ascorbate, or the reverse titration with hydrogen peroxide, showed that the "blue" copper has an E' value of 0.045-V lower than that of Cu(330) at 25°C (pH 7.0) (17). The two types of copper appear to be in redox equilibrium, but it is not certain that Cu(614) and Cu(330) interact intramolecularly. Our finding that Cr(II) reduces Cu(330) about 30% faster than Cu(614) is in accord with the above-mentioned data. The simplest explanation for the biphasic curves found for the reduction of laccase, followed either at 614 or 330 nm, is heterogeneity of the laccase preparation. Fungal laccase preparations have been shown to be heterogeneous from the EPR spectra of Type-2 copper (18). The protein has not been resolved into separate entities. The similarity of our results at 614 and 330 nm for *Rhus* laccase implies that the effect of the heterogeneity is felt by both types of copper. Why the heterogeneity is not observed at 330 nm at lower temperatures is not readily understandable.

The rate of chromous ion reduction of the "blue" copper in laccase is slower than reduction rates observed for the other copper proteins by factors of 10^2–10^4. Comparison of activation parameters for the laccase and spinach plastocyanin reductions reveals a dramatic difference in both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. A possible explanation for this behavior is that reduction of Cu(614) by Cr(II) takes place intramolecularly only after some Cu(330) has been reduced. Thorough investigation of the kinetic order with respect to Cr(II) should be helpful in discussing this possibility, as a mechanism involving intra- (or inter-) molecular electron transfer steps may not yield a simple experimental (Cr^{2+}) dependence. Experiments with ions inhibiting reduction of Cu(330) potentially could show if inhibition of Cu(330) affects the reduction of Cu(614) and provide an estimate of the rate of direct reaction, if any, between chromium(II) and Cu(614) in laccase.

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